Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7825–7829, September 1991
Genetics

Identification of a germ-line mutation in the p53 gene in a patient with an intracranial ependymoma

(inherited mutation/familial cancer syndrome/recessive oncogene)

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Communicated by Ira Herskowitz, June 24, 1991 (received for review April 3, 1991)

ABSTRACT **We detected a germ-line mutation of the p53 gene in a patient with a malignant ependymoma of the posterior fossa. This mutation, which was found at codon 242, resulted in an amino acid substitution in a highly conserved site of exon 7 of the p53 gene; the same mutation was found in both the germ-line and the tumor tissue. This is the most common region of previously described somatic p53 mutations in tumor specimens and of the germ-line p53 mutations in patients with the Li–Fraumeni cancer syndrome. Evaluation of the patient's family revealed several direct maternal and paternal relatives who had died at a young age from different types of cancer. The association of a germ-line p53 mutation with an intracranial malignancy and a strong family history of cancer suggests that p53 gene mutations predispose a person to malignancy and, like retinoblastoma mutations, may be inherited.**

Tumor-suppressor genes, recessive oncogenes that are associated with a variety of human cancers (1), were initially detected by restriction fragment length polymorphism (RFLP) analyses showing deletion of specific chromosome sequences (2). Recently, studies have shown both deletion and mutation of the p53 gene (human gene symbol, *TP53*) on chromosome 17 in several different types of tumors (3, 4). These and related findings (5, 6) have established that the p53 gene has tumor-suppressor activity like that of the retinoblastoma gene (*RB*), the prototype of a recessive oncogene. Whereas inherited and somatically acquired mutations of *RB* were initially identified in specimens of peripheral blood as well as tumor tissues from patients with retinoblastoma (7), mutations of the p53 gene were shown primarily only in tumor specimens (4). Recently, germ-line mutations of the p53 gene have been detected in affected members from families with the Li–Fraumeni cancer syndrome (8, 9).

We have detected a germ-line mutation in the p53 gene in a boy with an intracranial malignant ependymoma, a type of brain tumor not previously reported to be part of the Li–Fraumeni series of tumors (10). The same mutation was found in both the germ-line and the tumor tissue. This patient also had a strong family history of cancer in both the maternal and paternal kindreds, suggesting that the p53 gene mutation may underlie the etiology of his tumor.

MATERIALS AND METHODS

Molecular Genetic Tumor Studies. The patient, a 5-year-old boy, presented with headache, nausea and vomiting, and unsteady gait that had progressively worsened over a period of several months. A contrast-enhanced computerized tomography scan showed a large mass arising from the floor of the fourth ventricle. The patient underwent excision of the tumor mass. Pathologic examination of the tumor specimen showed features consistent with a low-grade ependymoma. Although the patient also received postoperative radiation therapy, the tumor recurred 2 years after surgery. A second operation was performed, at which time specimens of peripheral blood (20 ml) and tumor tissue were obtained following protocols previously approved by the Human Subjects Committee, Stanford University Medical Center. Pathologic examination of these tumor specimens showed features of a high-grade malignant ependymoma, consistent with the relatively rapid regrowth of the mass. His condition was stable for approximately 3–4 months, when the tumor recurred, ultimately resulting in his death.

Leukocytes from the peripheral blood were separated by centrifugation at 5000 × *g* for 15–20 min. The tumor specimen was stored at −80°C and was disaggregated manually at the start of the experimental procedure. Genomic DNA was extracted by published procedures (11). To determine whether there was a deletion of DNA sequences in the tumor specimen, RFLP analysis was performed using Southern blot hybridization techniques (11, 12). We selected the following probes from chromosome 17p because of our earlier results showing deletion of DNA sequences from this chromosome in medulloblastoma, another pediatric tumor of the posterior fossa (12): 144D6 (17p13.3), YNZ22.1 (17p13.3), HRP5.5 (17p13.1), Hp53b (p53 gene–17p13.1), EW 503 (17p12), MYH2 (17p12), and HF12-2 (17p12) (Howard Hughes Medical Institute Gene Mapping Data Base, Yale University, 1990). For electrophoresis, following digestion of the DNA, samples from the leukocytes and the tumor were run in adjacent lanes and the blood and tumor specimens were compared to determine any difference in their respective hybridization patterns for each of the probes. Absence of a band in the tumor tissue that was seen in the leukocytes from the same patient was scored as a deletion. The results were confirmed by densitometric analysis (12).

DNA amplification by means of the polymerase chain reaction (PCR) was used in combination with screening of the reaction products by denaturing gradient gel electrophoresis (DGGE) to study the blood and tumor specimens for p53 mutations. PCRs were performed using each of four pairs of oligodeoxynucleotide primers that were synthesized from published sequence data to cover the entire evolutionarily conserved region of the p53 gene (13). The primers were modified by the addition of a 40-base-pair G+C-rich sequence ("G·C clamp"), which had previously been shown to improve the sensitivity of the DGGE technique (14–16). The

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Abbreviations: RFLP, restriction fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis.

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primers** for exon 7 that proved to be informative were AOG 3 (5'-CGCCCGCCGCGCCCCGCCCGGCCCGCCGCCCCCGCCCGGGATCCGTGTTGTCTCCTAGGTTGGCT-3'; G·C clamp at 5' end) and AOG 4 (5'-GCCGGAATTCCAAGTGGCTCCTGACCTGGA-3'). The PCR was performed using previously described variables (17).

DGGE was performed with gel conditions and an optimal time of electrophoresis that were determined empirically based on previous studies (16). The PCR samples were prepared for DGGE by ethanol precipitation and resuspension in 30 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3 M NaCl. The samples were then denatured at 95°C for 5 min and were reannealed at 58°C for several hours, after which 10 μl of each sample was added to 10 μl of orange G loading dye and loaded on a DGGE apparatus. Electrophoresis was performed at 160 V on a parallel gradient gel containing a linear gradient of DNA denaturants ranging from 35% to 65% [100% denaturant = 7 M urea with 40% (wt/vol) formamide] at a constant temperature of 60°C for 8 hr. The gels were then stained with ethidium bromide and photographed. The presence of a novel band and heteroduplex bands on the gels indicated a gene mutation (14–17).

Multiple isolates of the p53 gene defined by the primers AOG 3 and 4 were subcloned and sequenced by standard methods (18, 19). A modified bacteriophage T7 DNA polymerase (Sequenase, United States Biochemical) and [α-[^{35}S]thio]dATP were used for the sequencing reactions. The four termination-reaction mixtures were then heated to 75°C for 2 min and loaded on an 8% polyacrylamide gel containing 7 M urea. Electrophoresis was then performed at 60-W constant power for 3–6 hr, after which the gel was dried for 1 hr at 80°C and exposed to Kodak XAR-5 film for 36 hr. The sequence for the fragment of the p53 gene defined by the primers was read from the gel and was compared with the published data for the p53 gene (7).

Family Studies. Historical information obtained from the patient's maternal and paternal relatives included the sex of family members, their current age or age at death, history of a cancer diagnosis, and cause of death. Diagnoses of cancer were verified with an examination of records and pathologic specimens whenever possible. In addition, molecular genetic studies were performed on peripheral blood specimens from paternal family members who were available for venipuncture. Early death from cancer of many younger relatives on the maternal side of the kindred limited the available data. No maternal relatives were available for molecular genetic analyses, nor were we able to obtain tissue blocks from those who had died. The family members studied were those identified in the pedigree as III-K; IV-J, -K, -L, and -M; and V-D, -E, and -F (Fig. 1). Peripheral blood (20–30 ml) was obtained from each person, and DNA was extracted as described (11). The DNA was then used for PCR amplification of exon 7 of the p53 gene followed by screening by DGGE.

DNA obtained from the blood of the available paternal relatives was analyzed to investigate the possible inheritance of the p53 gene mutation shown in the patient. Southern blots were prepared and hybridized with probes from chromosome 17p. In an attempt to identify the pattern of inheritance of the p53 gene region of chromosome 17p for the patient, a comparison was made of the allelic fragments obtained after hybridization for each informative probe tested against the DNA from each family member.

RESULTS

RFLP studies showed preservation of both alleles of all of the informative chromosome 17p probes tested against the DNA from the patient's leukocyte and tumor specimens. In particular, no deletions of the p53 gene were detected. The combined PCR–DGGE analysis, however, showed a mutation at codon 242 in exon 7 of the p53 gene in both the blood and tumor tissue of the patient. The mutation was detected on the denaturing gradient gel by the presence of a novel band and novel heteroduplex bands (Fig. 2*A*). The patient was heterozygous for the p53 mutation in the peripheral blood. Sequencing of the p53 exon 7 fragment confirmed this result (Fig. 2*B*). The mutation is a substitution of an adenine for a guanine, resulting in the replacement of a cysteine residue by a tyrosine residue. The site of the mutation in exon 7 is highly conserved across species (Fig. 3) and is one of the most common sites of p53 mutation in many types of neoplasms (4, 20) as well as in the blood and tumor tissue of affected family members with the Li–Fraumeni syndrome (8, 9).

No p53 gene mutations were detected in the DNA from available paternal family members who were screened with the combined PCR–DGGE technique. The chromosome 17p markers tested against those same DNA specimens gave no information to resolve whether the mutation was inherited or arose spontaneously. The lack of either fresh or archival tissue from the maternal family members therefore precluded verification of the origin of this germ-line p53 gene mutation.

DISCUSSION

We analyzed the p53 gene because previous studies have shown mutations in a variety of tumors in which deletions of chromosome 17p are well documented (3, 4), findings that project an important role of the p53 gene in human neoplasia (4, 20, 21). Studies such as those documenting the transformation of early-passage rodent cells by the p53 gene in cooperation with the *ras* oncogene suggested that the primary activity of the p53 protein was growth stimulation (5, 22). Subsequently, however, it was determined that those initial investigations were performed with a mutated version of the gene (5). The wild-type p53 protein has since been shown to be growth-suppressive, most recently in transfection experiments performed in colon cancer and glioblastoma cell lines (23, 24).

Although studies of breast cancer biopsy specimens that overexpressed a mutated p53 protein in a tumor-specific fashion suggested a role for the gene in human neoplasia (5, 25), a significant role for mutant p53 proteins was shown by molecular genetic analysis of colorectal cancer (3, 26). Deletion of the region of chromosome 17p containing the p53 gene was first detected with RFLP analysis in a large percentage of colorectal cancer specimens (26). Thereafter, PCR-based amplification studies of the p53 gene in colorectal cancer detected frequent point mutations, both in specimens that, with RFLP analysis, showed deletion of p53 alleles and, later, in the specimens in which both p53 alleles appeared to be preserved (3).

Our study shows a germ-line p53 gene mutation in a patient who developed an intracranial ependymoma. The mutation was detected in one of the four regions where p53 mutations are clustered ("hot spots"), sites that have been observed in colorectal, breast, lung, and adult brain tumors and that correspond to highly evolutionarily conserved exons of the p53 gene (4, 20, 21). Although definitive proof of altered gene function resulting from a mutation requires an assay of cellular transformation, the identification of a germ-line p53 mutation provides strong circumstantial evidence for its role in the etiology of our patient's tumor. At present, the mechanism by which mutation of the p53 gene results in neoplastic transformation has not been established. Transgenic mice produced from mutant p53 gene constructs show a phenotype consisting of several different kinds of tumors (6). Studies using a temperature-sensitive mutant p53 con-

**Primer sequences for the other p53 regions are available from P.H.C. at the Department of Neurological Surgery (Box 0112), University of California, San Francisco, CA 94143.

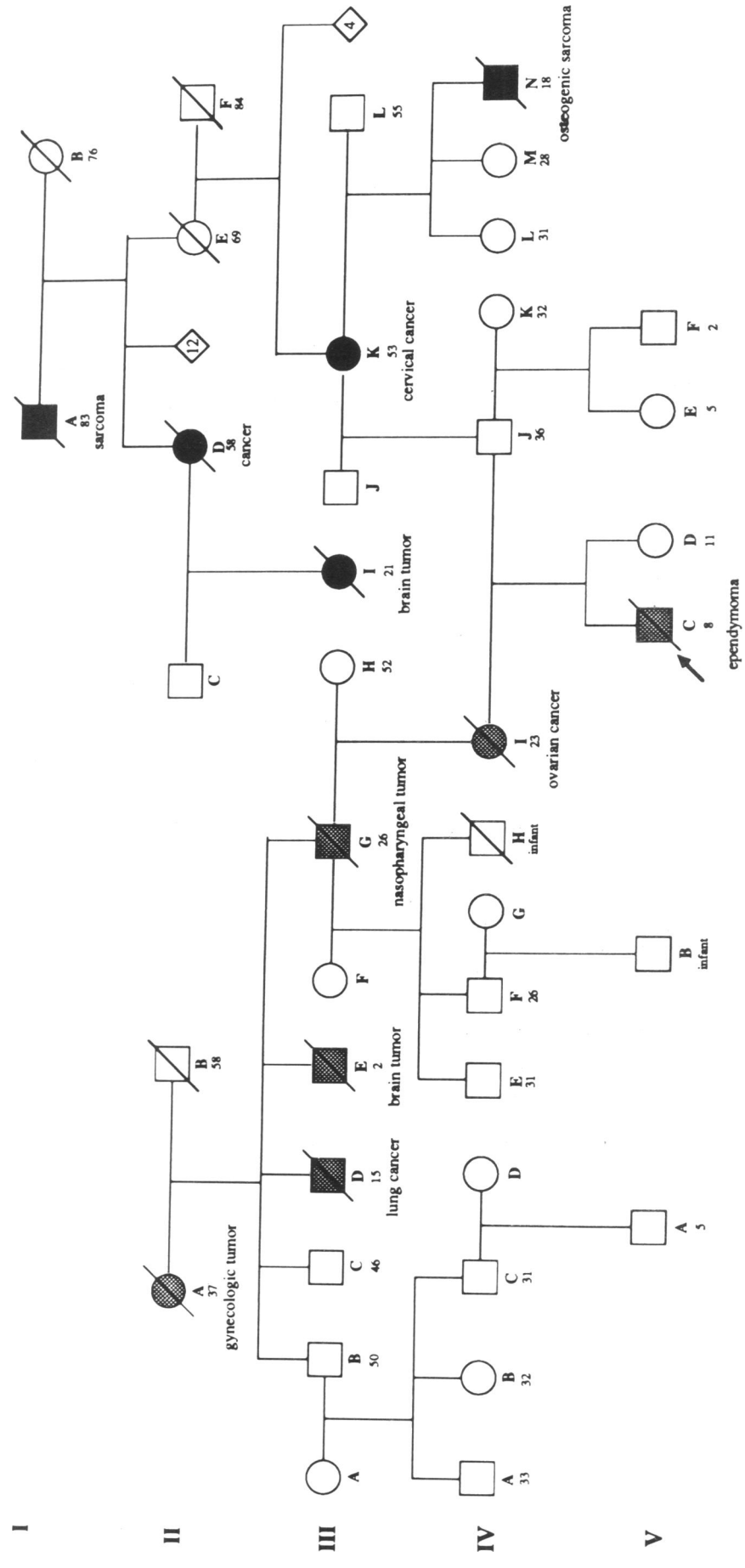

FIG. 1. Pedigree showing a high incidence of cancers in the kindred of a patient with germ-line p53 mutation. Black symbols, affected paternal relatives; stippled symbols, affected maternal relatives; diamonds, offspring of unknown sex. Numbers below symbols represent either current age or age at death (slash through symbol). Arrow indicates proband.

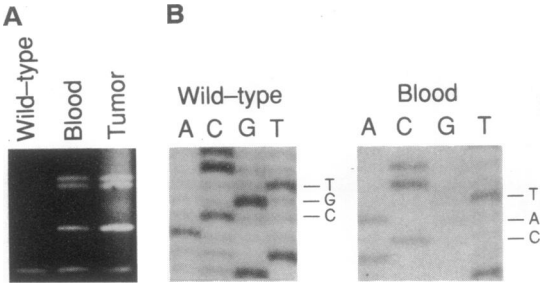

FIG. 2. (*A*) Denaturing gradient gel shows a germ-line p53 mutation. Novel band and novel heteroduplex bands are seen in DNA from blood and tumor specimens. (*B*) Sequencing gels showing wild-type and mutant p53 sequences at codon 242. A, adenine; C, cytosine; G, guanine; T, thymine.

struct have shown that the cellular proliferation and transformation induced by the gene is lost when the permissive temperature is raised and is restored when the temperature is lowered. This finding suggests that p53 activity may be reversible (27). A strong transcription-activating sequence in the amino-terminal part of the protein has also been detected, suggesting that p53 may activate an inhibitor of cellular proliferation genes (28).

Several familial cancer syndromes have been reported in which there is a high incidence of brain tumors associated with other types of neoplasms (29). In Li–Fraumeni syndrome, there is a predisposition to sarcomas, leukemias, breast and colon cancers, and gliomas of the brain (10), and affected individuals have been shown to harbor germ-line mutations of the p53 gene in exon 7 (8, 9). Although the members of our kindred did not show an increased incidence of the most common tumors in Li–Fraumeni syndrome, one of the family members (IV-N) developed an osteogenic sarcoma and several members (III-E, III-I, and V-C) had brain tumors. The detection of a p53 mutation in the family at exon 7 suggests that there is overlap of the family's pedigree with the more classic type of Li–Fraumeni syndrome. Moreover, as ependymomas of the brain are not typically part of the Li–Fraumeni syndrome, our results suggest that the syndrome may include other types of tumors.

The finding of a germ-line p53 mutation in the member of a cancer family kindred whom we describe, as well as the recent studies on several Li–Fraumeni kindreds (8, 9), suggest that, like mutations of *RB*, inherited mutations of the p53 gene can result in malignancy. Our results provide additional incentive to examine exon 7 of the p53 gene whenever Li–Fraumeni syndrome is suspected.

The PCR–DGGE combination technique allows rapid screening of DNA mutations in all types of tissue specimens. The specificity of the DGGE technique in detecting single base-pair mutations, particularly with the use of the G·C clamp, has been well documented (15, 16). The PCR requires

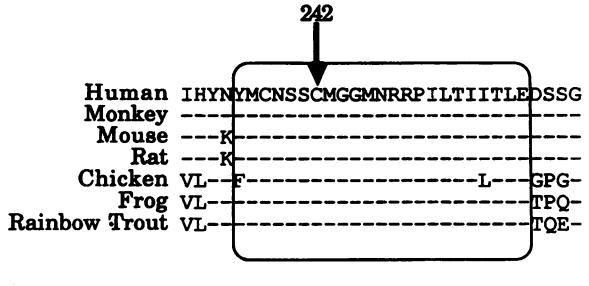

FIG. 3. Conservation of the site of germ-line p53 mutation across species. Amino acid sequences are shown with one-letter symbols. Dashed lines show sites of conservation; nonconserved sites are shown by the letters on the lines below the human sequence (see ref. 12).

only small amounts of genomic DNA, which can be obtained from materials ranging from needle biopsy specimens of tumor tissues to archival paraffin-embedded human specimens. Cancer family pedigrees can therefore be screened rapidly with the use of the PCR–DGGE technique for p53 and other tumor-suppressor gene mutations, provided that sufficient tumor tissues are available for study. We therefore hope that this work serves not only to identify a germ-line p53 gene mutation but also to act as a reminder to preserve archival specimens for such future investigations.

We thank the following individuals for providing probes: E. Wright, R. White, Y. Nakamura, D. Barker, B. Vogelstein, T. Takeuchi, N. Spurr, A. Ferguson-Smith, S. Weil, P. Chambon, M. Chao, E. Bakker, C. Buys, C. Schwartz, M. Litt, and R. Neve. We thank David Cox and Richard Myers for advice on the project and the reporting of the results, Susan Eastwood for editorial advice, and Cindy Huff and Cheryl Christensen for manuscript preparation. This work was supported by National Institutes of Health Postdoctoral Training Grant GM07085 and grants from the School of Medicine, University of California, San Francisco, and the National Brain Tumor Foundation. A.K.M. is a Howard Hughes Medical Institute Medical Student Research Training Fellow. G.D. was supported by Training Grant CA09291 from the National Institutes of Health.

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